

# Circulating biomarkers for early detection of hepatocellular carcinoma

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**Abstract:** Hepatocellular carcinoma (HCC) is estimated to be the fourth leading cause of cancer-related deaths worldwide. HCC patients face a dismal prognosis because symptoms usually appear in an advanced stage of disease. The detection of early stage HCC allows for curative surgical treatment and therefore saves lives. Specific non-invasive or diagnostic markers for HCC may represent a valuable tool for detecting these tumors at an early stage. The clinically most established serological biomarker alpha-fetoprotein shows only limited diagnostic performance, however novel candidate biomarkers and biomarker panels for detecting HCC at early stages of development are being studied. In this review we will discuss the findings of these studies.

**Keywords:** diagnostics, early biomarkers, early diagnosis, hepatitis B virus, hepatitis C virus, hepatocellular carcinoma, immunology, liquid biopsy, micro RNA, surveillance, viral hepatitis

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## Introduction

Hepatocellular carcinoma (HCC) is estimated to be the fourth most common cause of cancer-related deaths, and the second leading cause of years of life lost from cancer worldwide, with approximately 800,000 fatalities every year.<sup>1,2</sup> When individuals are diagnosed with HCC due to the presentation of symptoms, it is often at an advanced stage, leading to poor prognosis with limited treatment options.<sup>3</sup> The majority of HCC develops in cirrhotic livers, as the presence of cirrhosis is thought to provide a pro-carcinogenic intrahepatic environment.<sup>4</sup> Less than 10% of HCC develop in non-cirrhotic livers.<sup>5,6</sup> The most important risk factor for development of liver cirrhosis and liver cancer worldwide is chronic viral hepatitis caused by hepatitis B virus (HBV) or hepatitis C virus (HCV) infection, as well as alcohol abuse and a variety of metabolic conditions.<sup>7</sup> Non-alcoholic fatty liver disease (NAFLD), usually related to overweight and metabolic syndrome, is an emerging cause of HCC.<sup>8</sup> The shared hepatocarcinogenic effect of all these risk factors is thought to be the inflammation-driven damage mediated *via* the effects of reactive oxygen species, associated with telomere alterations

and chromosomal instability, leading to the development of HCC in individuals at risk.<sup>9,10</sup> Globally, the majority of HCC can be attributed to HBV infection.<sup>2</sup> The persistent virus-induced immune triggering response in the infected liver can lead to fibrosis and cirrhosis, which may eventually lead to HCC. In addition, HBV, and to a lesser extent NAFLD, can harbor 'direct' mechanisms of liver carcinogenesis in the absence of cirrhosis. In the case of HBV, viral-mediated hepatocarcinogenesis has been suggested to be related to viral integration into the host genome inducing both genomic instability and direct insertional mutagenesis of diverse cancer-related genes.<sup>11</sup>

Early detection of HCC is critical for a curative approach as tumor cure is only feasible when detected at a small size. Thus, screening for HCC is recommended in those at risk in order to detect early, small tumors. Currently, individuals at risk are advised to undergo ultrasonography of the liver every 6 months with the optional addition of measuring alpha-fetoprotein (AFP) in blood. Ultrasound has acceptable sensitivity of 65–80% for HCC detection and has an upper level of specificity of more than 90%.<sup>12,13</sup> However,

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tumor size and body habit significantly affect the sensitivity of ultrasound in detecting HCC. Sensitivity ranges from 42% for lesions smaller than 1 cm to 90% for tumors of larger size such as those in advanced stage HCC. Early stage tumors are smaller and thus more difficult to detect, particularly in patients with nodular cirrhotic livers or obesity. Beyond its sensitivity for nodule detection, ultrasound screening represents a rather cumbersome process for patients. Despite the non-invasiveness of the test, patients need to be fasting for the procedure and it demands medical appointment times. In addition, ultrasound is dependent on operator expertise, not always yielding the same level of sensitivity or specificity. Therefore, routine HCC screening by ultrasound is often not implemented properly making patients with HCC more likely to be diagnosed at late stages.<sup>14–16</sup> Detection of early stage HCC has a more favorable disease prognosis, because patients are more likely to benefit from tumor resection, liver transplantation, or tumor ablation.<sup>17</sup> In order to reduce morbidity and mortality from HCC there is a clear need for non-invasive, quantifiable biomarkers that identify the early stages of HCC, thereby allowing the implementation of more efficient and cost-effective surveillance strategies. An ideal biomarker for early HCC detection must fulfill certain criteria: it must be specific for HCC, minimally invasive to detect, simple to process, cost-effective, and superior to currently used HCC biomarkers. It must have good detection performance [sensitivity; specificity; area under the receiver operator curve (AUC)] and yield consistent results across genders, different ethnic groups, and underlying liver diseases. Numerous studies have been conducted that evaluate a broad range of novel biomarkers in blood for their ability to detect and predict the early stages of HCC. However, only a few have achieved enough accuracy to be recommended as optional by international societies.<sup>18–21</sup> In this review, we will discuss the findings of studies that we believe represent the most advanced biomarkers and report their performance for detecting early stage HCC.

#### **AFP, AFP-L3, Des- $\gamma$ -carboxyprothrombin and the GALAD model**

AFP is the only serological biomarker which is clinically used as a diagnostic and prognostic marker for HCC and recommended by some international guidelines.<sup>18–21</sup> However, the potential of AFP in

the early detection of HCC is sub-optimal as serum levels show a wide variation in sensitivity and specificity due to elevated levels of AFP in disorders, such as viral hepatitis, cholangiocarcinoma, metastatic colon cancer and other tumors.<sup>22</sup> Application of AFP as a biomarker for determining HCC before the actual HCC diagnosis by imaging has been examined.<sup>23,24</sup> When determined up to 12 months before visual confirmation, the sensitivity using an AFP cut-off of  $>20$  ng/ml was only 3%, whereas a cut-off of  $\geq 200$  ng/ml resulted in a sensitivity of 43%.<sup>23,24</sup> Although the performance of AFP has low sensitivity and specificity, clinical practice may still benefit from the use of this marker as it can improve the diagnostic sensitivity of ultrasound for early stage HCC compared with the use of ultrasound alone.<sup>24,25</sup>

Lectin-binding AFP-3 (AFP-L3) is a glycoform of AFP, and the ratio of AFP-L3 to total AFP has also been reported as a candidate serological biomarker for HCC. An AFP-L3 ratio higher than 35% increases the specificity to 100% for HCC in patients with serum AFP levels of 10–200 ng/ml.<sup>26</sup> Moreover, some studies have shown AFP-L3 levels to be increased in patients 3–18 months before the tumor is detectable *via* imaging.<sup>24,27</sup> A recent study by Choi *et al.* reported that 6 months prior to detection by imaging, the combination of AFP-L3 and AFP levels was able to detect HCC with a sensitivity of 66% and a specificity of 85%.<sup>24</sup> Interestingly, even 12 months before HCC could be detected by imaging, the combination of the two serum markers had a sensitivity of 55% and a specificity of 81% to detect early stage HCC. These numbers were obtained in a study in predominantly chronic HBV patients with cirrhosis.<sup>24</sup> In the same study, des- $\gamma$ -carboxyprothrombin [DCP, also known as prothrombin induced by vitamin K absence-II (PIVKA-II)], was also included for the detection of early stage HCC. DCP is a non-functional prothrombin produced by HCC.<sup>28,29</sup> It was previously described to improve the diagnostic performance of AFP, and is included in the Japanese guidelines since 2015 for HCC surveillance and diagnosis, as one of the components of an algorithm.<sup>30</sup> A Japanese study that included 1377 patients at all stages of HCC concluded that DCP was not superior to AFP in detecting small tumors, but it performed better than AFP for larger HCCs.<sup>31</sup> A study by Lok *et al.* with 24 early stage HCC patients showed that DCP complemented AFP levels in detecting HCC up to

12 months before the tumor was visible by imaging in HCV associated HCC (sensitivity and specificity of 73% and 71% at month -12, and 86% and 69% at month -6, respectively).<sup>23</sup> Importantly, Choi *et al.* showed that combining DCP, AFP, and AFP-L3 did not improve the surveillance performance over AFP and AFP-L3 alone.<sup>24</sup> These studies were well designed and the difference in outcome underlines the complexity of HCC heterogeneity.

A further sophistication of the use of the three biomarkers (AFP, DCP, AFP-L3) for detection of early stage HCC came with the inclusion of gender and age as additional parameters into a prediction model, called the GALAD model.<sup>32</sup> The GALAD score was validated and compared with ultrasound in a large multicenter cohort of HCC patients with various underlying diseases, like HBV and HCV infections or alcohol abuse.<sup>33-35</sup> In subgroup analyses of patients with early HCC, Yang *et al.* reported that the sensitivity and specificity of the two methods were comparable (sensitivity GALAD 0.92 *versus* ultrasound 0.92, and specificity GALAD 0.79 *versus* ultrasound 0.79).<sup>33</sup> The diagnostic performance of the GALAD score to detect early stage HCC was validated in large trials performed in the United Kingdom (sensitivity 80%, specificity 90%), Japan (sensitivity 82%, specificity 82%), Germany (no data reported due to small sample size) and a multi-center cohort of the National Cancer Institute Early Detection Research Network (EDRN) in the USA (79% sensitivity, 79% specificity).<sup>33,36</sup> The performance of the GALAD model for detecting early stage HCC is good and it may even detect HCC before detection by imaging. The addition of age and gender makes it outperform the combination of the serological markers AFP, AFP-L3, and DCP. Inclusion of ultrasound readings in the model (GALADUS) further improves the performance for early HCC detection with a sensitivity of 88% and a specificity of 94% in the EDRN cohort.<sup>33</sup>

### Protein biomarkers for early detection of HCC

In an effort to further improve the early detection of HCC many studies have evaluated the performance of other highly diverse candidate protein biomarkers. However, thus far, none of these candidate HCC biomarkers have been adopted in clinical practice or recommended by large

professional hepatology societies, underlining the complexity and challenges faced in biomarker development. However, different approaches aimed at discovering proteins more abundantly expressed by tumors than by normal tissue resulted in the identification of a number of promising candidate molecules that have been suggested as potential biomarkers for early stage HCC, as will be discussed in the following.

Glypican-3 is one of the proteins that has been found to be highly expressed by the HCC tumor, and can also be detected in serum.<sup>37</sup> A recent meta-analysis reported that this transmembrane protein could not discriminate HCC from liver cirrhosis patients, however it did improve the utility of AFP in diagnosing late stage HCC.<sup>38</sup> Insufficient data is available on whether circulating glypican-3 can be used to detect early stage HCC.

The search for novel candidate HCC biomarkers has also made use of the knowledge acquired from other types of cancer research; such biomarker proteins include squamous cell carcinoma antigen (SCCA) and antibodies against SCCA,<sup>39-41</sup> osteopontin,<sup>42-46</sup> Golgi protein 73,<sup>47</sup> Heat shock protein 27,<sup>48</sup> Dickkopf-1,<sup>49</sup> anti-Ku86,<sup>50,51</sup> lamin B1,<sup>51</sup> vimentin,<sup>51,52</sup> aldo-keto reductase family-1 member B10 (AKR1B10),<sup>53</sup> and fucosylated kininogen.<sup>54</sup> These markers have been explored for the diagnosis of early stage HCC. The circulating proteins anti-Ku86, osteopontin, Dickkopf-1, and Golgi protein 73 have been shown to outperform the sensitivity of AFP for detecting early HCC<sup>43,45,47,55,56</sup> but no large trials have validated these results, and, to our knowledge, only osteopontin has been examined for detection of HCC prior to their detection by imaging. A pilot prospective study, including 22 Asian patients who developed HCC during follow-up, detected elevated osteopontin levels 12 months before diagnosis by imaging.<sup>43</sup> The same research group continued their study in a large heterogeneous cohort of Europeans, and found that the combination of osteopontin and AFP identified patients at high-risk for HCC up to 2 years before diagnosis.<sup>57</sup> An interesting and recent multicenter study from Asia demonstrated that AKR1B10 serum levels detected early stage HCC with a sensitivity of 61% and a specificity of 86% for discriminating early stage HCC from chronic hepatitis B and liver cirrhosis controls. The combination of AKR1B10 with AFP showed

a comparable diagnostic performance (62% sensitivity and 87% specificity).<sup>53</sup> Although these results were exciting, the early stage HCC cohort was relatively small, and therefore it is important to reproduce these findings in a larger cohort of Asian patients, as well as in non-Asian patients.

The fact that no single protein is able to accurately diagnose or predict the future development of a tumor illustrates the complexity and heterogeneity of HCC development. This process of hepatocarcinogenesis is further marred by different tumor-produced proteins in different etiologies of liver disease, as well as inflammatory biomarkers differing according to the underlying liver process that originally led to HCC. Most studies compare the performances of AFP levels with the novel candidate biomarker proteins. However, comparable with the GALAD model, another study aimed to improve their existing HCC detection model of AFP, age, gender, alkaline phosphatase, and alanine aminotransferase by the addition of fucosylated kininogen. This panel of parameters demonstrated an impressive AUC of 0.97 (95% confidence interval 0.94–1.00) in a subgroup of 113 patients (29 early stage AFP-negative HCC *versus* 84 cirrhotic patients).<sup>54</sup> Cross-validation of this interesting data is needed as these multi-parameter statistical models require large sample sizes to account for overfitting.<sup>58</sup> In conclusion, the above-mentioned candidate biomarkers are still in experimental phases and currently no evidence has been provided that they surpass the utility of ultrasound or the GALAD model in diagnostic performance. Large biomarker studies are needed to show whether these markers complement existing diagnostics.

### Inflammatory biomarkers for early detection of HCC

One can also envisage that dysplastic lesions consisting of transformed liver cells modulate the micro-environment of the liver at early stages of the process, resulting in quantifiable changes in certain markers. Examples of events that are likely to occur are the release of bio-active molecules in dysplastic or small tumor lesions that modulate the behavior and secretion of surrounding cells in a paracrine manner (Figure 1). In addition, recognition of the tumor cells by leukocytes followed by activation and infiltration of other immune cells is a likely scenario. This process to sense transformed cells is known as immunosurveillance

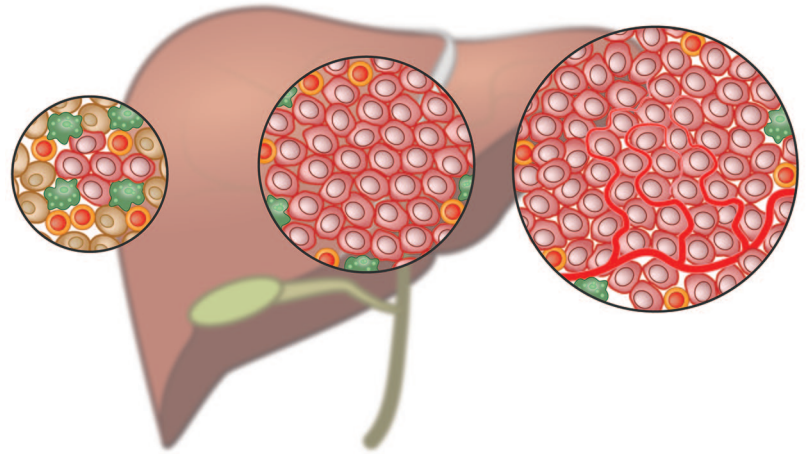
and has been well-described for many tumors. As a consequence, cytokines and chemokines induced upon recognition of the dysplastic or cancerous lesion may be found in serum or plasma.

In a recent study, we identified a series of immune markers detected in plasma of patients with HCV that could predict the development of *de novo* HCC, even when some cases occurred up to 2 years after blood collection, in subjects who were negative by ultrasound at the time of blood sampling. This immune signature was markedly different from matching controls comprising HCV patients who did not develop HCC in the same time period. The serum immune markers included, among others, soluble proteins such as monokine induced by interferon-gamma (MIG), interleukin (IL)-22 and IL-3, which are immunomodulatory molecules, as well as vascular endothelial growth factor (VEGF) and TNF related apoptosis inducing ligand (TRAIL), which are related to vascular formation and apoptosis modulation.<sup>59,60</sup> Interestingly, this subsequent change in immune parameters could be quantified regardless of the ongoing inflammation secondary to HCV infection. The utility of TGF- $\beta$  as a potential biomarker for HCC development has also been reported; serum levels of TGF- $\beta$  are associated with HCC development<sup>61</sup> and were reported to predict the development of HCV-associated HCC in a subgroup of patients.<sup>62</sup> No data is available on the possible use of serum TGF- $\beta$  as a biomarker for early stage HCC.

### MicroRNA and epigenetic markers for early detection of HCC

MicroRNAs (miRNA) are non-coding RNAs of about 22 nucleotides in length that function as regulatory RNAs for both natural and malignant processes. MiRNAs can be detected in serum as well as in vesicles, and numerous studies have determined the utility of miRNA serum levels for the detection of a variety of tumors.<sup>63</sup> MiRNAs regulate up to one-third of the cellular functions *via* RNA silencing and post-transcriptional regulation of gene expression,<sup>64</sup> and are also involved in controlling processes, such as liver fibrosis, cell differentiation, and tumorigenesis.<sup>65</sup> During early cancer development, abnormal miRNA expression occurs and certain miRNAs can be detected in blood in various forms, such as micro-vesicles.<sup>66,67</sup> Moreover, as miRNAs are very stable and tolerant to extreme temperature and pH





<b>Performance of HCC biomarkers in:</b>	<b>Pre-cancer</b>	<b>Early stage</b>	<b>Advanced stage</b>
<i>Order of importance of biomarker producing cells</i>	Hepatic stellate cell > Lymphocytes > Malignant cells	Malignant cells > Hepatic stellate cells > Lymphocytes	Malignant cells > Hepatic stellate cells > Lymphocytes
<b>AFP, AFP-L3, DCP and the GALAD model</b>	Not validated	Validated in large multicenter trials	Validated in large multicenter trials
<b>Protein markers</b> <i>(i.e. Glypican-3, Golgi protein 73, AKR1B10)</i>	Not well studied	Not validated	Not validated
<b>Inflammatory markers</b> <i>(Interleukins and angiogenic factors)</i>	Not validated	Not validated	Not validated
<b>MircoRNA</b> <i>(i.e. microRNA-122, microRNA-21)</i>	Not validated	Not validated	Not validated
<b>Epigenetic markers</b>	Not well studied	Not validated	Not validated

**Figure 1.** Hepatocellular carcinoma (HCC) development and corresponding diagnostic utility of well-known and novel circulating biomarkers for detection of precancerous HCC, early stage HCC and advanced stage HCC.

levels,<sup>68</sup> they are suitable for detection in various bodily fluids, such as serum and urine. MiRNAs are measured by polymerase chain reaction, whereas protein biomarkers are generally detected by immunoassay or mass spectrometry.

Studies on miRNA for accurate HCC detection have shown that panels of multiple miRNAs<sup>69–71</sup> have better diagnostic performance than a single miRNA.<sup>72–75</sup> The majority of these studies included predominantly Asian individuals with chronic HBV infection, and only a few studies report on their utility for early HCC detection. One such study (in 166 HCC patients) identified a panel comprised of miR-122-5p, miR-100-5p, miR-125b-5p, miR-885-5p, and miR-148a-3p, that differentiated early stage HCC from healthy individuals, but not from those with risk factors

like viral hepatitis or cirrhosis.<sup>71</sup> Only one study assessed whether circulating miRNAs were able to detect HCC prior to detection by imaging. This retrospective study detected HCC 12 months before imaging with a low sensitivity, but with a high specificity (30% and 85%, respectively) and measured the following miRNA panel: miR-29a, miR-29c, miR-133a, miR-143, miR-145, miR-192, and miR-505.<sup>76</sup> Although miRNAs offer potential for early detection, few studies found a consistent set of miRNAs that correlate with early stage HCC in multiple studies. Only miRNA-122 and miRNA-21 – both dysregulated in liver diseases – have been reproduced so far in different studies.<sup>71,77–79</sup> To appreciate the utility of miRNA for early HCC detection, standardization of the assays, improved sensitivity, and cross-validation in all etiologies are needed.

Recently, epigenetic markers have also been included in studies as candidate biomarkers for HCC. A study in the United States with 95 HCC patients and 51 cirrhotic controls showed that a methylated DNA marker panel had a diagnostic sensitivity of 75% for solitary HCCs smaller than 2 cm and a sensitivity of 93% for solitary early stage HCC smaller than 5 cm. The panel examined the homeobox A1, empty spiracles homeobox 1, AK055957, endothelin-converting enzyme 1, phosphofructokinase, and C-type lectin domain containing 11A genes, normalized by beta-1,3-galactosyltransferase.<sup>80</sup> In addition, the discovery of highly chemically stable 5-hydroxymethylcytosine proved an interesting tool for measuring circulating cell-free tumor DNA.<sup>81</sup> A Chinese group identified a panel of 32 genes implicated in HCC, HBV, or hepatic fibrosis, and they used this in a weighted model to differentiate HCC from chronic hepatitis or liver cirrhosis.<sup>82</sup> In their validation cohort of 220 early HCC *versus* 129 controls, the diagnostic model achieved 83% sensitivity and 67% specificity in distinguishing early stage HCC from control patients with HBV or liver cirrhosis.<sup>83</sup> Genetic markers are an attractive technique as they potentially detect disease specific components, such as circulating tumor DNA. Such markers reflect cancer specific traits and may guide clinical practice. However, these assays are still only applied in the research field, and standardization of this technique is needed. Furthermore, the sensitivity and specificity of genetic markers will have to improve before validation in global trials.

### Concluding remarks

Surveillance markers that detect curable HCC are needed. A marker that detects tumors earlier than a 6-month period before the tumor is visible by imaging allows for early curative therapy. Moreover, it will identify patients who are not at risk and thus do not need close monitoring. Such a marker will reduce mortality, discomfort, and cost. Current surveillance programs rely on ultrasound, which has an acceptable sensitivity for more advanced stages of HCC. However, it may take years for a liver nodule to progress to a radiologically “visible” HCC<sup>84</sup> and detection of early stage tumors is unsatisfactory, particularly in patients with nodular cirrhotic livers or obesity. Also, HCC screening by ultrasound is often not implemented in areas with limited resources.<sup>14,85</sup> A cost-effective and non-invasive biomarker for

early HCC detection is therefore essential. It will have to perform consistently across gender and ethnic groups and must not be affected by the presence of common risk factors of HCC, like cirrhosis or fulminant hepatitis virus infection.

AFP is a poor predictor of early HCC development; however, when combined with age, gender, AFP-L3, and DCP (GALAD model) it comes of age, shown in Figure 1. This robust statistical model displays good performance, in various etiologies of HCC, for early stage HCC and it potentially detects HCC prior to detection by imaging.<sup>33</sup> A disadvantage, which is common to all serologic approaches to early diagnosis, is that although the marker may suggest that an HCC has developed, the actual diagnosis is made by imaging and thus confirmation is required for further clinical management. Data on the utility of other protein markers in surveillance is lagging behind; this is true for the candidate protein markers like glypican-3, but also immune markers, miRNA, and epigenetic markers, and it is likely that in the years to come further optimization of the biomarker panels for early HCC will be reported. One way to improve the performance of a biomarker is by increasing its sensitivity by making use of so-called liquid biopsies. This non-invasive approach utilizes the detection of biomarkers in bodily fluids, such as tumor fragments (circulating cell-free DNA, miRNA, nucleic acids) circulating tumor cells, and excreted vesicles containing proteins and genetic material.<sup>86–88</sup> These markers have the advantage of being tumor specific, and may reflect the intratumoral heterogeneity and evolution that we normally cannot detect *via* other serological markers. Ideally, HCC is detected *via* tissue biopsy, however, HCCs are highly vascularized and even fine needle aspirates can induce tumor hemorrhaging.<sup>89</sup> Others consider sampling error or needle tract seeding of HCC cells as important limitations.<sup>90</sup> These issues are not relevant when collecting liquid biopsies.

Identification of a new biomarkers for early detection of HCC is clearly complicated by the heterogeneity of the tumors as well as the diverse patient populations. The heterogeneity caused by different etiologies, involvement of environmental factors (such as aflatoxin), but also genetic factors, as shown by the identification of susceptibility single nucleotide polymorphisms for HCC, need to be taken into account in biomarker studies.

Their performance will need to be validated in appropriate clinically relevant control groups with biopsy-proven diagnosis and in prospective cohorts of representative size and statistical power. Candidate markers require international validation using standardized methodology that allow for global adoption of these techniques in clinical practice. Furthermore, new markers can deepen our understanding of the pathogenesis of HCC, further classify HCC subtypes, and identify novel treatment targets.

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### Conflict of interest statement

The authors declare that there is no conflict of interest.

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